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PATENTS

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

In re application of
Jean Plouet et al.
Serial No. 09/091,561 GROUP 1644
Filed August 21, 1998 Examiner G. Ewoldt
ANTI-IDIOTYPIC ANTIBODIES OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND USE THEREOF AS DRUGS

Rule 132 Declaration of Dr. Jean Plouet

I, Jean Plouet, hereby declare as follows :

I am the same Jean Plouet named as an inventor in the above-identified patent application. My relevant background and experience are set forth on the attached c.v. I make this declaration in support of the present application, and to provide evidence in rebuttal of several contentions in the Official Action of May 10, 2000, that various claims of this patent application would have been obvious from the applied prior art.

In 1989, we had prepared polyclonal antibodies in rabbits against murine VEGF purifies as described in Plouët et al, EMBO J, 1989. These antibodies proved to be neutralizing for the binding of iodinated VEGF to endothelial cells and for the proliferation and migration of endothelial cell induced by VEGF.

Since it was impossible to decipher the functions mediated *in vivo* by each VEGF receptor because (i) VEGF does not circulate (ii) the VEGF receptors were not identified, we decided to construct circulating agonists of VEGF receptors, in order to :

- purify and characterize VEGF receptors,
- decipher the biological functions exerted by each VEGF receptor.

In winter 1992, we injected subcutaneously these anti-murine VEGF antibodies to rabbits, and therefore raised anti-idiotypic antibodies described in the abstract published in the Journal of Cellular Biochemistry, January 4-23, 1994 ("*the 1994 Abstract*").

The properties that we discovered about these antibodies are that :

- they acted as agonists of the VEGF receptors,
- they induced proliferation and migration,
- they were not internalized.

At the time of the filing of this patent application, it had been demonstrated that VEGF binds to vascular endothelial cells on two distinct binding sites, one with a Kd of 2-10 pM and another one with a Kd of 50-100 pM (Plouët, Biochimie, 1990 ; Plouët, J Biol Chem, 1990). However, it is not clear, even today, that one site could correspond to one receptor, especially when considering the differences between *in vivo* and *in vitro* situations. We also demonstrated that VEGF was able to bind to non endothelial cells such as lens epithelial cells or corneal endothelial cells on a single site with a Kd of 5-10 pM. These non-endothelial cells did not proliferate under VEGF addition, but migrate. Similarly, we showed that "*In vivo*, VAS/VEGF (VAS, for vasculotropin, was the previous name of VEGF) was recognized as an inducer of angiogenesis and vascular permeability. *In vitro*, despite a moderate action on proliferation, VAS/VEGF strongly stimulates the cell migration" (Favard,...., Plouet et al, Bio Cell 1991).

Our interpretation at this time, and is still true today, was that this production of anti-idiotypic antibodies was not specific of a single VEGF receptor, even though we had hoped that, among so far unknown VEGF receptors, one should mediate migration, another one proliferation. The VEGF receptor flt-1 had been cloned in 1992 (De Vries, Science 1992), with indications that flt-1 was expressed in endothelium during neovascularization of healing skin wounds and during early vascular development in mouse embryos. The receptor KDR and its murine homologue flk-1 had been released to public knowledge only in 1993 (Quinn et al, Proc. Natl. Acad. Sci., 1993), at the time of the submission of *the 1994 Abstract* to the January 1994 Keystone Symposium. In this Quinn 1993 publication, it was demonstrated that "VEGF and its receptors flk-1 and flt-1 may play a role in vascular development and regulation of vascular permeabilty". At that time, our own various publications were

all confirming that our hope of different specific exclusive functions for flt-1 and flk-1 was not proven :

- Bovine Retinal Endothelial Cells (BRECs) bind VAS-VEGF on two high-affinity binding sites (apparent Kd of 2 and 56 pM) and can proliferate and migrate upon the addition of recombinant VAS-VEGF. (Simorre-Pinatel,..., Plouet et al, Invest Ophtalmol Vis Sci 1994).
- *In vitro*, despite a moderate action on proliferation, VAS/VEGF strongly stimulates cell migration. *In vivo*, VAS/VEGF is a potent inducer of angiogenesis and vascular permeability. (Plouet et Bayard, Horm Res 1994).

We thought that we had obtained anti-idiotypic antibodies recognizing both VEGF receptors known at this time, flt-1 and KDR/flk-1, in accordance with most published findings (cf Annex 1) which did not assign one single function to each receptor. We also thought that these anti-idiotypic antibodies recognized also other growth factor receptors such as those of FGF2 (Fibroblast Growth Factor-basic form or PDGF (Platelet Derived Growth Factor). The latter was based on the following:

It has been established that subcutaneous injections of highly basic synthetic peptides such as the one encoded by the exon 6 of the long form of PDGF can release endogenous PDGF, which behaves as an auto-antigen, and thus elicit antibodies directed against the exon 6 of PDGF and other sequences of PDGF. The genes of VEGF and PDGF have exactly the same sequences of exon 6; but these sequences are aligned in reverse senses in both genes. This means that eliciting antibodies against VEGF might raise antibodies against VEGF and PDGF. (Raines and Ross, J.Cell.Biol 1992). We were also working of this topic and established that synthetic peptides of exon 6 of VEGF could release endogenous FGF2 from extracellular matrix. These findings were published later on (Jonca,..., Plouet, J.Biol. Chem 97).

From the results published in *the 1994 Abstract*, we believed, and still do, that the anti-idiotypic antibodies obtained might represent internal images of not only VEGF but also FGF2 released from extracellular matrix.. So, we decided to interrupt this strategy : we had been looking for antibodies

which could help us understand and discriminate between the functions of VEGF receptors. We found ourselves trapped with antibodies inducing both functions, proliferation and migration.

We abandoned this research aimed at finding a true correlation between each receptor and a specific function in angiogenesis, an idea which, even today, is less and less likely to be true. We continued our research on another function of VEGF : In previous years, we had demonstrated with Praloran (Praloran,…,Plouet, CRAS, 1991) that VEGF induced the proliferation of lymphocytes, and thus that VEGF was a true interleukine.

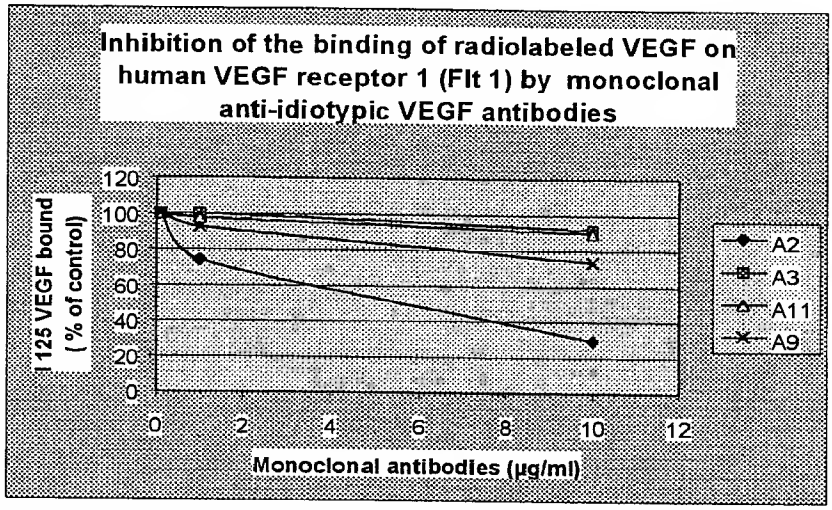
One of the roads we wanted to follow was to induce a vaccination by priming lymphocytes (especially dendritic cells) against VEGF, using anti-idiotypic antibodies of VEGF, hopefully circulating internal images of VEGF, as antigens. But we had to be sure not to produce versatile images, as it seemed to have been the case with the sub-cutaneous immunization.

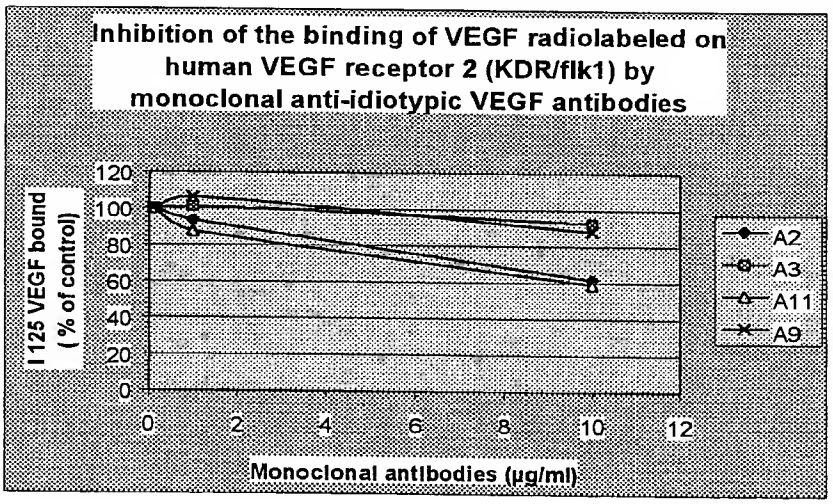
F. Jonca, a surgeon resident in urology, was working in our laboratory on the role of VEGF in prostatic adenocarcinoma. We chose with him and Praloran to immunize rabbits by injecting VEGF in lymph nodes, hoping to get thus another set of antibodies richer in anti-VEGF. We injected again these antibodies in lymph nodes of other rabbits and we started to analyze the functions of these anti-idiotypic antibodies.

We transfected COS cells with pSV 7d expression plasmids (Quinn et al, PNAS 93) containing the coding sequences of flt-1 and flk-1 and measured the inhibitory activity of anti-idiotypic antibodies towards the binding of iodinated VEGF to these cells, in order to discriminate between true anti-idiotypic antibodies of VEGF and other antibodies representing internal images of other growth factors as the ones we think we obtained during our previous research of a correlation receptor-function.

The results demonstrated that, unexpectedly considering our previous failure to get to specific exclusive functions for each receptor, one of these anti-idiotypic

antibodies was able to bind to KDR/flk-1 but not to flt-1, and to induce cell proliferation but not migration.





A 96 well plate was coated overnight at 4°C with 1.8 µg/ml of anti human Fc (Jackson Immuno research) in 0.05M carbonate buffer pH 9.6, then saturated 2 hours at room temperature with 0.05M carbonate pH 9.6, 0.5% BSA and further incubated with 100ng/ml of either human Fc chimera Flt 1 or human Fc chimera KDR (R&D) 3 hours at room temperature in the same buffer containing 0.05% tween (PBS-TB). Monoclonal antibodies were incubated overnight at 4°C in 100 µl of PBS-TB containing 0.5 µg/ml heparin and 125 pg of iodinated VEGF165. The wells were washed three times with PBS-TB and bound VEGF was removed from plates with 0.5M NaOH and the radioactivity was counted in a gamma counter Packard Cobra. The non specific binding was determined with an excess of unlabeled VEGF (100 ng/ml) and A3 represents an isotype control. The results are expressed as the percentage of specific binding, computed as follows:

cpm observed - non specific binding / cpm observed in the absence of antibody - non specific binding.

From this immunization of a mouse with the same polyclonal neutralizing antibody anti-VEGF, we derived several

monoclonal anti-idiotypic antibodies. In this figure we show the data corresponding to a set of four monoclonal antibodies of the same isotype. A3 does not bind to any of these two receptors, A9 binds to flt1, and does not bind to flk1. A11 binds to flk1 but not to flt1. A2 binds to both flt1 and flk1.

As a conclusion, we can summarize the following :

1. At the time of the filing of this patent application, a person skilled in the art had learned from most published publications that proliferation and migration (or differentiation) could be induced by the same ligands, or their internal images such as anti-idiotypic antibodies, since there was no clear knowledge of a specific function for each receptor. Conversely, a person skilled in the art following the few publications linking flk-1 with proliferation and flt-1 with migration, would have deducted that the anti-idiotypic antibodies described by *the 1994 Abstract,* which induced proliferation and migration, were ligands of both flk-1 and flt-1.

2. Although KDR/flk-1 and flt-1 have 50% overall homogeneity, they display 100% homogeneity in some clusters of aminoacids in their extracellular domain :

2.1 It is well admitted that the minimal size of a proteic epitope corresponds to 5-6 contiguous aminoacids or to longer stretches with a single aminoacid difference. If one compares the extracellular domains of KDR/flk-1 (SWISSPROT accession number P35968) and flt-1 (GENPEPT accession number X51602) it appears that sequences 241-247 and 506-511 of KDR/flk-1 are 100% homologous to sequences 246-252 and 511-516 of flt-1. Furthermore sequences 178-184, 300-306 and 695-701are 85% homologous to 183-189, 305-311 and 700-706 of flt-1.

2.2 It was not clear for someone skilled in the art at the time of the invention, and even today, that KDR/flk-1 is the only receptor responsible for the angiogenic (proliferation, migration and differentiation) process. To blur a concept already not so clear about the functions of each receptor, many publications referred to the "differentiation process", including extracellular matrix digestion, migration, proliferation, and cell organisation in order to form tubes, when showing functional results about the interaction of VEGF

with its receptors. Obviously, other receptors than KDR/flk-1 are involved in the proliferation process.

Patent US5952199, filed as late as June 13, 1997 after the filing of our patent application, from Davis-Smith et al cites in the background of the invention that :

"Recent gene knockout studies have demonstrated that both the flt-1 and KDR receptors are essential for the normal development of the mammalian vascular system, although their respective roles in endothelial cell proliferation and differentiation appear to be distinct. Thus, the endothelial proliferative and angiogenic activity of the VEGF protein is mediated by binding to the extracellular ligand-binding region of the flt-1 and KDR receptors on the surface of vascular endothelial cells."

2.3 *The 1994 Abstract* teaches that anti-idiotypic antibodies of VEGF bind to VEGF receptors. Established data cited above show that these antibodies had certainly to bind to KDR/flk-1 and to flt-1 to induce proliferation and migration. It is highly probable that other receptors known since, such as neuropilin-1 (Soker et al, Cell, 1998) were involved in the growth rate of the prostatic tumor. It has to be reminded that neuropilin-1 is not only expressed by tumor endothelial cellls, but also by prostatic cancer cells. Thus, it would not have been *prima facie* obvious for a person skilled in the art to deduct that the antibodies raised would be more likely binding to KDR/flk-1, than to flt-1, neuropilin or other receptors unknown at the time.

2.4 The present invention shows that it is possible to discriminate among antibodies binding to the two receptors KDR/flk-1 and flt-1, some antibodies binding only to one of these receptors. The present invention claims the production of antibodies binding only to KDR/flk-1, and not to flt-1.

2.5 It was not clear for someone skilled in the art at the time of the invention, and even today, that KDR/flk-1 is the only receptor responsible for the angiogenic (proliferation, migration and differentiation) process. Other

receptors are involved. There was no clear evidence linking a specific function to a specific receptor.

2.6 The present invention is the first ever to produce means to activate with no doubt one receptor without activating the other ones. Thus, the contributions of each receptor to each function (proliferation, migration, and differentiation) can be studied and used.

3 The way the antibodies described in *the 1994 Abstract* were produced is different from the production from the present invention: VEGF was injected in subcutaneous way in *the 1994 Abstract* antibodies, whereas the antibodies of the present invention are produced by injecting VEGF in lymph nodes. This difference is essential since we can suppose today that subcutaneous immunization against VEGF certainly produced antibodies against VEGF but also other antibodies against FGF and other growth factors epitopes, in particular epitopes corresponding to the binding domains of various growth factors for their receptors.

4. Internalization of the complex " KDR/flk-1 and the claimed antibodies " is the most important property of the antibodies of the invention, which was not taught by *the 1994 Abstract*.

5. Kieber-Emmons teach that Fab fragments and anti-idiotypic antibodies should exert the same immunogenicity and therefore would equally protect an animal against the same antigen. It is not challenged that vaccines using Fab fragments or the whole anti-idiotypic immunoglobulin would lead to the same vaccinal efficacy.

This purpose is fully different from our claim for bivalent antibodies which can dimerize the receptors of VEGF whereas Fab fragments, which are monovalent, exert an opposite function in endothelial cells : they can only block the VEGF receptors and, in no case, can induce a function similar to that exerted by the anti-idiotypic antibody, namely, receptor dimerization, internalization and cell proliferation.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further

that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Jean Plouet

Date : September 22, 2000